STUDIES ON STAPHYLOCOCCUS AUREUS WITH REFERENCE TO COAGULASE ACTIVITY

by

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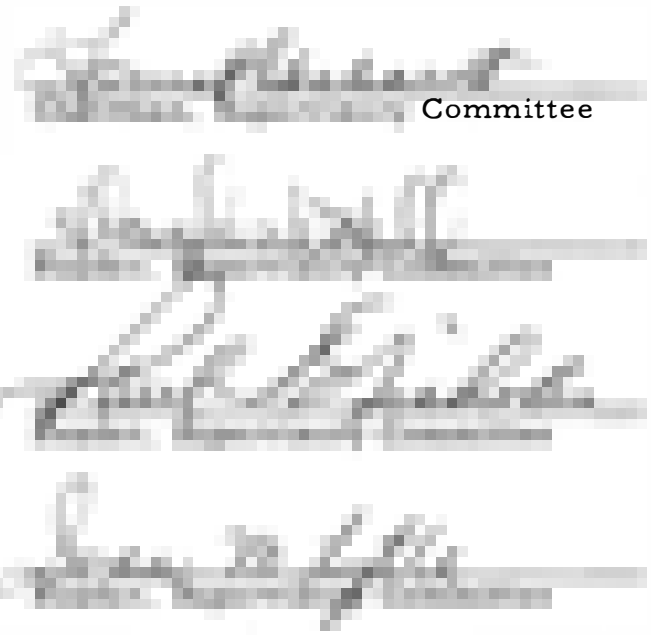

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INTRODUCTION

Virulent staphylococci are known to elaborate metabolic products (enzymes and toxins) which have been implicated in the virulence mechanism of these organisms; however, a predominating role cannot be assessed to any single product.

Production of coagulase by strains of *Staphylococcus aureus* has customarily been taken as an *in vitro* reaction indicating potential virulence.

Marks (1952) suggested that production of staphylococcal alpha toxin to be a more accurate indicator of virulence than is coagulase activity. Hoeprich et al. (1960) stated that tellurite reduction was of the same significance in regards to determination of potential pathogenicity as was coagulase.

Ekstedt and Yotis (1960) found that the virulence of coagulase negative staphylococci could be enhanced by suspending the organisms in solutions of partially purified coagulase; staphylococcal filtrates free of coagulase activity but not of alpha hemolysin were lethal to suckling mice (Gebhardt et al., 1962).

Extensive investigations relating coagulase and alpha hemolysin with the virulence of staphylococci have been carried out. Common

occurence of these factors in strains of known pathogenicity suggests that they are significant in the virulence of Staph. aureus.

Implication of the role of the metabolic products elaborated by staphylococci is evident; however, the direct demonstration of the role of any one factor in the pathogenesis of these organisms has not been made.

These studies were carried out in an attempt to determine the role of coagulase in the virulence mechanisms of Staph. aureus.

LITERATURE REVIEW

I. HISTORICAL

Pasteur in 1880 was able to culture in broth small spherical organisms from the pus of suppuration. Upon inoculation of the culture into rabbits formation of an abscess took place and micrococci could be isolated from the abscess. He also observed micrococci in pus from osteomylitis and was able to culture them in broth.

Ogston (1881) investigated eighty-two abscesses and was able to demonstrate the presence of cocci in each; however, the same staining methods never revealed them in tissues which were free from suppuration. Upon injection of pus containing cocci into animals, septicemia and local suppuration appeared. He was also able to grow staphylococci in the albumen of eggs, and these cultures were capable of producing lesions in animals even after they had been diluted.

He also linked the yellow pus with the color of the growth of staphylococci and noted that this yellow pus was an indication of a dangerous form of infection. It was noted by early investigators that light enhanced the appearance of the pigment and that heat and repeated cultivation on artificial media decreased the amount of color (Kolle and Otto, 1902).

Lubinski in 1894 noticed that when these organisms were cultured anaerobically less color was produced.

Rosenbach (1884) classified the yellow or golden pigmented forms of staphylococci as Staphylococcus aureus and the nonpigmented forms as Staph. albus. However, pigmentation as a basis of classification proved to be unsatisfactory because of frequent bacterial variation (Bigger et al., 1927).

Early attempts to classify these organisms was directed mainly toward a division of the staphylococci into two main groups: those pathogenic to man and animals, and those which occurred as commensals and elsewhere in nature. However, staphylococci are too widely distributed in nature to resort to animal experimentation in each case; therefore, evidence was sought to differentiate Staph. pyogenes as the pathogenic group became known (Fleming, 1929) from the type found on the skin named Staph. epidermidis albus by Welch (1891). Use of tests for bacterial variation suggested by Winslow et al. (1920) and use of precipitin tests suggested by Dudgeon (1908) produced evidence that both the white and golden pathogenic strains belonged to the same species.

Kolle and Otto (1902) were the first to attempt serological classification and claimed that agglutination tests with immune serum could differentiate between the pathogenic and saprophytic strains. Dudgeon

and Simpson (1928) concluded that there was no difference between the orange and white pathogenic strains. Walker and Adkinson (1917) found that immune serum prepared against strains of S. aureus agglutinated orange strains but not white ones and vice versa. None of these procedures came into general use for identifying strains.

Although the largest percentage of reported data concerning staphylococci has been obtained employing animals, some direct virulence tests in man have been carried out. Garre (1885) was first to test staphylococcal cultures on man. He scarfied the nail-bed of his left hand and infected it with a platinum wire direct from the colony obtained from a fatal case of osteomyelitis; however, no infection developed. In his second experiment he rubbed an entire slope culture into the skin of the left forearm. This caused a burning sensation in six hours and small pustules appeared around the hair folicles in about 12 hours. These enlarged the next day and within a week developed into a large carbuncle.

Elek in 1956 studied the dose required to elicit reaction in human beings. He found that upon intradermal injection of doses less than a million staphylococci, freshly isolated from pyogenic lesions, led to a little swelling and some transient redness, but no further ill effects developed. When the dose was increased to about seven million organisms, a small postule resulted in each of five volunteers, while the same dose given as a heat-killed control produced only redness in the same subjects.

He concluded that the smallest dose leading to pus formation can be accepted for purposes of comparison as a relative measure of virulence since it represents the smallest number of cocci capable of overcoming the body defense-mechanism, at least temporarily, and of multiplying in the tissues. That actual multiplication is required was shown by the fact that killed cocci in the same dose failed to result in pus formation. He also noted that the preformed toxin carried over from the broth culture made little difference in the results. The minimum pus-forming dose was the same with washed organisms, although the lesions were somewhat less red and swollen. Mixing of smaller numbers of cocci with larger amounts of toxin failed to produce pus, although the toxin by itself caused extensive and painful swelling. The mixing of the staphylococci in less than the pus-forming doses with plasma obtained from the same volunteers never set up a progressive lesion leading to pus. Results obtained from the study of 20 human volunteers were consistent: the smallest pus forming dose was about 1 to 5 million cocci. Three strains obtained from lesions and nine strains from nasal carriers were compared in pairs of volunteers and no clear differences in virulence could be demonstrated between the two groups of staphylococci.

II. CHARACTERISTICS OF STAPHYLOCOCCUS AUREUS

A. Virulence Factors.

Blair (1962) divided the virulence factors into two categories: (1) those which enable the staphylococci to invade the host and infect (leukocidin, hyaluronidase, coagulase), (2) those which affect or damage the tissues (hemolysins or toxins). The two "virulence factors" that have received by far the most attention are coagulase and alpha hemolysin, which is probably due to the common occurrence of these substances associated with strains of known pathogenicity.

According to Blair, 1962, coagulase is of primary importance to the staphylococci only in the very early stages of infection, that is during the time in which the organisms are trying to establish themselves in the tissues. Once they become established the role of coagulase becomes less important. Menken and Walston (1935) suggested that the staphylococcal lesion was due to the exotoxins produced during the metabolic activities of the organisms.

A great deal of work has been done, but as of yet, no single factor has been incriminated as the sole indicator of virulence. The virulence of S. aureus depends upon many physiological factors. Staphylococci produce many diffusible products which have been implicated in varying degrees in the pathogenicity of staphylococcal infections.

According to Burns and Holtman (1960) a virulent staphylococcus is one that is obtained from a typical pyogenic abscess in pure culture and that it may also spread from its favored subcutaneous sites to other tissues where it may give rise to pneumonia, septicemia or osteomyelitis.

B. Hemolysins.

At the present time no definite statement can be made regarding the relationship of the alpha hemolysin to pathogenesis. However, due to the frequent production of the alpha hemolysin by strains obtained from lesions, one is lead to the conclusion that it must either play a major or minor role in the pathogenicity of Staph. aureus. As with many phases of staphylococcal virulence, considerable controversy exists concerning the role of alpha hemolysin in the pathogenicity of Staph. aureus.

A close correlation exists between the production of alpha hemolysin and the production of coagulase. Many investigators have confirmed this fact (Chapman et al. 1934; Bryce and Roundtree, 1936; Cowan, 1938). Gillespie, Devenish, and Cowan (1939) found that all coagulase-positive strains produced alpha lysin and similar findings were repeated by Christie et al. (1946). Schwabacher et al. (1945) found that 91 per cent of coagulase-positive strains studied produced alpha hemolysin. Marks (1952) tested one hundred strains of Staph. aureus and found that all of them produced alpha hemolysin and concluded that the production

of alpha hemolysin was a more accurate and convenient criterion of the pathogenicity of staphylococci than the coagulase test.

Contrary to the above finding Elek and Levy (1954) concluded that the detection of alpha hemolysin production was neither as convenient nor reliable as coagulase testing as a measure of the pathogenicity of staphylococci.

In studies by Lack and Wailling (1954) neither the production of coagulase, fibrinolysin nor hemolysin could be accurately correlated with pathogenicity. They showed in their survey that only eighty-two per cent of the 435 strains examined produced alpha hemolysin. They concluded as did Elek and Levy (1954) that alpha hemolysin was not an absolute criterion of pathogenicity.

Two other types of hemolysins are know to exist in staphylococcal filtrates. Glenny and Stevens (1935) demonstrated a second serologically different type called beta hemolysin. While investigating the correlation between tube hemolysin tests and appearances on blood agar, Williams and Harper (1947) observed a third hemolysin and called it the delta hemolysin.

Morgan and Graydon (1936) claimed to have shown the existence of two alpha hemolysins, $alpha_1$ and $alpha_2$. Smith and Price (1938) reported a hemolysin which they called gamma hemolysin. Elek and Levy (1950) reported that evidence is strongly indicative that the gamma hemolysin is identical to the delta hemolysin. It must be borne in mind that it is a

difficult task to analyze the many claims because of the fact that several methods and techniques have been employed in the demonstration of these various hemolysins.

C. Staphylococcal Hyaluronidase.

Staphylococcal hyaluronidase has been studied extensively. Schwabacher, et al. (1945) studied the production of hyaluronidase and found that of the coagulase-positive strains 93.6 per cent produced hyaluronidase, while no enzyme was produced by the strains that didn't produce coagulase. Duran-Reynals (1933) studied fifty-three strains of staphylococci and concluded that invasive properties paralleled the existence of a spreading factor in their filtrates. He injected rabbits on one side with staphylococcal cultures and on the other side they injected filtrates from the same strain mixed with India ink, the size of the lesion compared closely with the size of the area in which the ink had spread.

Under conditions of in vitro culture, hyaluronidase appeared early and generally increased in amount for the first six days of incubation Davison et al. (1949). Rogers (1953, 1954) compared the rate of liberation of hyaluronidase into the medium during growth, with rate of liberation of coagulase and with increase in numbers of cocci. He found that coagulase was liberated first at the time when growth started, but increased at a slower rate than growth; on the other hand

when hyaluronidase production was compared with multiplication, there was a lag period during which no hyaluronidase was produced followed by a period in which hyaluronidase production was produced at a greater rate than growth of cells.

It is very difficult to assess the role of hyaluronidase in the virulence of staphylococci. Some investigators claim a relationship between hyaluronidase and virulence while others claim there is no causal relationship.

D. Leucocidins.

Another metabolic product of staphylococci that has been implicated in the virulence of staphylococci is leucocidin. Van de Velde (1894) observed that the pleural exudate produced by the injection of virulent staphylococci had a destructive effect on rabbit leucocytes. In 1900 Neisser and Wechsberg showed that a strong toxin would kill polymorphonuclear leucocytes within two minutes and that it was lost upon storage and suggested that hemolysin and leucocidin were separate substances.

These early observations were based on the effect of leucocidin upon rabbit white cells. However, Panton and Valentine (1932) used known leucocytes and found that a factor in staphylococcal toxin is capable of inhibiting their phagocytic ability. This leucocidin became known as the "Panton-Valentine leucocidin" to distinguish it from the one described by Neisser and Wechsberg. The Panton-Valentine leucocidin acts on both human and rabbit leucocytes whereas the one described by Neisser and

Wechsberg acts only on rabbit leucocytes and appeared to be identical with alpha hemolysin.

More recently Gladstone and van Heyningen (1957) showed that two leucocidins exist, in addition to the Neisser-Wechsberg leucocidin. One of these is the Panton-Valentine leucocidin and the other is what they refer to as a "leucolysin" which acts on leucocytes of all species with the exception of sheep. Leucolysin has many properties in common with the delta lysin and is produced by a large number of coagulase positive staphylococci and by some coagulase negative strains.

E. Capsules.

Early investigators have indicated that Staph. aureus does not normally possess a capsule, but Lyons (1937), claimed to have demonstrated capsules in very young cultures, within the first three hours of growth. The capsules disappeared after 24 hours incubation but Spink (1939) was unable to confirm these findings.

Kleineberger-Nobel (1948) was also unable to demonstrate the presence of capsules in various non-mucoid strains of staphylococci. There are, however, references in the literature to the occurrence of strains of Staph. aureus which possess large capsules and form mucoid colonies (Gilbert, 1931). These were usually isolated from human lesions.

Price and Kneeland in 1954 obtained a capsulated Staph. aureus by passage of a non encapsulated strain through an embryonated hen's egg. This varient was identical on phage typing with the parent strain and showed no difference in virulence. Later attempts to isolate this variant failed; however, these investigators in 1956 were able to demonstrate by use of a capsular swelling reaction that almost all coagulase positive staphylococci formed small amounts of capsular material.

III. PROPERTIES OF STAPHYLOCOCCAL COAGULASE

The ability of certain bacterial species to clot plasma was first reported in 1903 by Loeb. Much (1908) and Gross (1931) investigated the clotting of human plasma by a specific substance produced by staphylococci called staphylocoagulase.

Despite rather widespread acceptance of staphylococcal coagulase as an index of pathogenicity, the mode of action of this substance and its significance in vivo remain hypothetical. Many theories have been reported as to its mode of action.

Duthie (1954) recently reported the existence of two different kinds of coagulase. One a soluble coagulase which is liberated into the medium causes clotting of plasma (fibrinogen) and the other a bound coagulase causes clumping of the organisms by plasma or fibrinogen alone. This stimulated Kapral (1960) to determine if either kind of coagulase is itself an essential virulence factor.

This was done by selecting a strain of Staph. aureus known to be virulent for rabbits and possessing both kinds of coagulase. Mutants lacking either kind of coagulase were then derived from this strain and checked for virulence in rabbits. He isolated three mutants, one lacking the soluble type of coagulase and the other lacking the bound type; these were both virulent for rabbits. The third mutant which produced both kinds of coagulase was identical biochemically and physiologically with the parent strain except for loss of virulence.

Much (1908) working with seven strains of staphylococci, obtained from lesions and a number obtained from other sources, came to the conclusion that only pathogenic strains produce coagulase, but the first systematic studies linking coagulase production with pathogenicity were made by Daranyi (1925, 1926, 1927) who compared the established criteria of pathogenicity with coagulase production and found the correlation valid. Gross (1931) confirmed Daranyi's technique and also noted that many Staph. albus produced coagulase also. Chapman, Berens, Peters, and Curcio (1934) suggested a combination of hemolysin and coagulase tests for identifying pathogenic staphylococcus, but more recently the coagulase test has gained acceptance.

Broadly speaking, coagulase may play one of two roles in the disease producing ability of staphylococci: it may have a part in the initiation of the lesion or it may protect the lesion already formed. It is well known

that in inflammation a network of fibrin is deposited early in the tissues and this together with the thrombosis of the lymphatics draining the region, leads to the establishment of a mechanical barrier to the passage of certain foreign particles. According to Menken (1931, 1933, 1935) the barrier helps to circumscribe the irritating substance and allows a definite period of time for the leucocytes to assemble for the purpose of phagocytosis. In this way vital organs are protected at the expense of local injury. The coagulase action seems to offer an easy explanation for the findings; however, the evidence is to the contrary. The fibrin barrier can be demonstrated by the injection of trypan blue when the inflammation is caused not only by staphylococci, but by other organisms. The injection of crude coagulase does not lead to the formation of the fibrin barrier, but a toxic filtrate of staphylococci will do so, showing that toxin and not coagulase is responsible in the natural staphylococcal lesion (Menkin and Walston, 1935). More attractive is the view that coagulase has a role in the initiation of staphylococcal lesions and in particular may impede the mechanism of elimination of the cocci (Delrez and Govaerts, 1918).

A. Coagulase Production.

The simplest chemically defined medium studied for the production of coagulase contained a wide range of amino acids and vitamins (Lominski et al. 1950). In this medium only a small amount of coagulase was produced and reached its maximum concentration after 3 to 5 days'

incubation. When staphylococci are grown in simple digest broth or in infusion broth, there is generally no difficulty in demonstrating the presence of coagulase after 24 hours' incubation.

Various authors differ in their views as to the length of time of incubation required for maximal coagulase production; however, it is rather difficult to make comparisons because different strains of organisms, different media and methods for detection of coagulase have been used. It must be borne in mind that strains and even individual colonies of the same strains vary in their ability to produce coagulase.

The addition of egg yolk or serum to media was found to stimulate the production of coagulase (Davies, 1951). It is possible that the presence of protein protects against a coagulase destroying factor (Lominski, Smith and Morrison, 1953). Duthie (1954) found that the presence of serum actually enhanced coagulase production, the important factor was found in the albumin fraction.

Conditions which are suitable for the production of other products of staphylococci are not necessarily suitable for the production of coagulase. It is known that incubation in an atmosphere of carbon dioxide favors production of alpha hemolysin, but is inhibitory to coagulase production (Di-Rocco and Fulton, 1939).

The amount of coagulase produced by different strains in vitro shows no correlation with the ability to cause infection. In other words a strain

that produces a large amount of coagulase is not necessarily more capable of producing an infection in man. Local lesions in man may be caused by staphylococci that exhibit varying degrees of coagulase activity. A great majority of strains obtained from lesions show low activity (Tager and Hales, 1947).

Lack (1956) stated that it is very likely that coagulase contributes to the offensive arsenal of staphylococci, but it would be unwise to assess its importance relative to other factors elaborated by them. At most its action is transient, saving the organism from phagocytosis during the initial phase and enabling it to multiply and produce toxins. Rogers and Tompsett (1952 a, b) have shown that although inhibition of phagocytosis does occur in human plasma when coagulase positive strains are used, the subsequent course of events may be of greater importance. They found that after an initial drop in the number of organisms there was a rise accompanied by destruction of the leucocytes. This did not occur with coagulase negative strains. The presence of fibrin had no effect, and toxic substances were responsible for the destruction of the leucocytes. There was a striking difference in the ability of staphylococci to survive after ingestion by leucocytes: coagulase negative strains were destroyed within three to four hours, while the coagulase positive strains survived.

B. Action of Coagulase Upon Plasma from Various Animal Species.

In all coagulase tests the first requirement is a suitable plasma. Since the action of coagulase is upon plasma, the plasma from many animal species have been studied. Those studied have been human (Walston, 1935; Pijoan, 1935) which is susceptible to the action of coagulase. The plasmas of horse (Richou 1949), dog (Walston, 1935) pig (Pijoan, 1935) and goose (Loeb, 1903) will also clot. Mouse plasma does not clot (Smith and Hale, 1944). Guinea pig plasma resists action of coagulase at 37° C but is susceptible at 20° C (Smith and Hale, 1944; Kaplan and Spink, 1948).

In most instances it was noted that the plasmas behaved irregularly and this irregular behavior of plasma of the same species suggested that an accessory factor might be required for the action of coagulase. This irregular behavior of the different plasmas, prompted Smith and Hale in 1944 to carry out experiments in attempts to demonstrate the existence of such a factor. They found that testicular extract was a suitable source of this accessory factor and that a cell-free preparation of coagulase would clot mouse and guinea pig plasma in the presence of the testicular extract which are not normally clotted by coagulase.

Further proof that testicular extract aided in the process of coagulation was obtained by incubating a mixture of coagulase and testicular extract and removing an aliquot of the mixture at different time intervals

and testing it on guinea pig plasma. A decrease in clotting time was noted. The end product was thermolabile and had a thrombin like action. It showed complete loss of activity at 56° C and partial loss at 45° C. The behavior of guinea pig plasma can then be explained on the basis that it contains very little accessory factor and at 37° C its combination with coagulase and the destruction of the product proceed simultaneously, whereas at 20° C the rate of formation of the resultant product exceeds the rate of destruction.

The evidence is conclusive that the coagulating principle is the result of interaction of staphylococcal coagulase and some factor or factors present in sera and in tissues. This coagulating principle is sometimes referred to as "coagulase-thrombin" which differs in various respects both from the accessory factor and staphylococcal coagulase. Both the accessory factor and staphylococcal coagulase are moderately heat stable, coagulase-thrombin is more labile.

Since the standardization of a batch of plasma on a large series of staphylococci is not practicable, testing each strain with a series of decreasing plasma concentrations has been suggested by Duncan and Walker (1942). Once a suitable plasma has been found satisfactory for use in coagulase tests, it can be preserved. If it is dried and stored at 4° C, it remains satisfactory for at least eleven months (Chapman, Berens and Stiles, 1941).

C. Techniques for Determination of Coagulase.

The principal value of coagulase to the medical bacteriologist is undoubtedly the fact that it forms the basis of a simple laboratory test for the recognition of Staph. aureus. The test involves the use of animal plasma, which is a variable material. There is good reason to think that although coagulase is a characteristic biological product of pathogenic strains, the amount produced even when conditions are standardized varies from strain to strain. Added to these difficulties is the fact that Staph. aureus produces fibrinolysin which is capable of destroying the clot or coagulum before a reading can be taken.

Broadly speaking there are three ways in which the production of coagulase can be detected. The first method is carried out by mixing a culture of staphylococci with plasma or decalcified blood in a tube and the visual observation of the clot. A second method depends on the cultivation of pathological material on nutrient agar containing plasma. The deposition of insoluble fibrin resulting from coagulase action appears as a zone of turbidity around the staphylococcal colony. The third method is based on the observation of Much (1908) that the addition of a suspension of staphylococci to plasma results in an immediate clumping of the organisms. This can be carried out on a slide; however, routine coagulase tests are usually carried out by adding equal volumes of a suitable plasma plus a suspension of staphylococci in a test tube and incubating the mixture at

37° C. Formation of a coagulum or clot is an indication of the presence of coagulase. Hoeprich et al. (1960) suggested that tellurite reduction was of the same significance with regards to determination of potential pathogenicity of staphylococci as is the tube coagulase test.

D. Chemical Nature of Coagulase.

It has been found that coagulase activity is always associated with protein. Statements that coagulase is dialysable (Walston, 1935) have not been confirmed (Tager, 1948). The results with gradocol filtration suggests that coagulase has an enormous particle size, but ultracentrifugation studies do not bear this out. High-speed centrifugation for three hours failed to reduce the activity of a highly purified preparation, although the same treatment sedimented poliomyelitis virus. Even the most highly purified samples of coagulase so far obtained are not electrophoretically homogeneous, there being at least two components of different mobility (Tager, 1948). The active material can be precipitated with alcohol, Walston, (1935) and Fisher, (1936) and so concentrated. Impurities may be precipitated from crude preparations by 5 to 20 per cent saturation with ammonium sulfate; half saturation and acid precipitation bring down the active material (Walston, 1935). These methods combined with ethanol fractionation yield a preparation of coagulase active at a dilution of one part in several millions. This material has a nitrogen content of 14 to 16 per cent, the amount generally found in proteins. The most

convincing evidence of the protein nature of coagulase is furnished by its behavior towards proteolytic enzymes. Crude coagulase is readily inactivated by both trypsin and pepsin. Purified coagulase is also inactivated by crystalline trypsin and chymotrypsin.

E. Filterability of Coagulase.

Coagulase freed of living cocci was first obtained by Gonzenbach and Uemura (1916) who noted that it was more resistant to heating than the organisms. In this way a crude coagulase preparation was obtained by centrifuging the culture and sterilizing the supernatant fluid by heat; as a result of this treatment considerable loss in activity occurred. This was due to removal of the bacterial cells, Gratia (1920).

Early workers found conflicting results when they tested the filterability of coagulase. Some found that Seitz filtration removed all activity Walston, (1935) while others did not find this to be so, Gross (1928), Fisher (1936). In 1944 Smith and Hale used gradocol membranes and found that coagulase passed freely through those with an average pore diameter of 0.5 u, activity was reduced after passage through those with average pore diameters of 0.31 u and disappeared on filtration through a membrane of average pore diameter of 0.11 u. However, the filterability of coagulase is affected by the pH of the culture, since coagulase produced in digest and extract broths is filterable through Seitz pads when the pH is adjusted to 6.7 or less (Lominski and Milne, 1947).

F. Action of Physical Agents.

Purified coagulase remains active for several months if kept in a desiccator at 5° C. Solutions maintained at 15° C to 20° C will also remain active for months but rapidly deteriorate at higher temperatures. They are most stable at a pH of 4.5 to 7 and deteriorate markedly under alkaline conditions. The relative resistance of coagulase to heat was noted early, but the findings are contradictory concerning the actual temperature resisted. It is generally agreed that crude coagulase is capable of withstanding 60° C for thirty minutes without appreciable loss of potency (Walston, 1935; Smith and Hale, 1944). Smith and Hale, 1944, found that a 120° C coagulase was destroyed in ten minutes, while others report that after autoclaving at 120° C for twenty minutes still yielded an active preparation (Walker, Derow, and Schaffer, 1948).

G. Action of Chemical Agents.

The action of a variety of substances on coagulase has been investigated. Storage of broth cultures of staphylococci in the presence of 0.15 per cent phenol, 0.1 per cent permanganate does not destroy coagulase, but 0.3 per cent formaldehyde at 37° C leads to complete inactivation within 48 hours (Gengou, 1933). Oxidizing agents are inhibitory, bromine in a concentration of 0.3 per cent or more and 0.8 per cent iodine both inhibit coagulation by Berkefeld filtrates of staphylococcal cultures (Farkas, 1947).

Coagulase reacts variably with reducing agents. Ascorbic acid is injurious, Thioglycollate exhibits no action whereas propylene glycol and sodium azide are inhibitory (Walker, Derow, and Schaffer, 1948). Sulfathiazole in concentrations of 1 to 10 mg. per ml is said to accelerate the clotting action of cell-free filtrates, and a similar effect was found with sulfanilamide and para-aminobenzoic acid by Spink and Vivino (1942). These workers found sulfadiazine to be strongly inhibitory, but Walker, Derow, and Schaffer in 1948 were unable to detect any inhibition due to sulphadiazine.

Of the antibiotics studied, streptomycin was found to inhibit the action of coagulase (Agnew, Kaplan and Spink, 1947). Erythromycin in concentrations below the inhibitory level was found to interfere with the activity of coagulase of living cultures. Similar results were obtained with tetracycline and chloramphenicol, but to a lesser degree (Boniece, Holmes and Wick, 1956). Bacitracin was found to be inactive against coagulase (Walker, Derow and Schaffer, 1948).

IV. VIRULENCE OF STAPHYLOCOCCI

The virulence mechanism of staphylococci even at best is still obscure despite the fact that many products elaborated by them have been implicated as virulence factors. There is a vast amount of literature dealing with these various products elaborated by staphylococci and their relationship to the virulence of these organisms.

Virulence by definition applies to a given host, and in the case of staphylococci, virulence is assessed in relation to man. However, in studying this organism it is neither practical nor possible to study them in relationship to their human host, although some studies have been carried out in human volunteers (Elek, 1956; Elek and Conen, 1957); so experimental data must be obtained by employing laboratory animals and extrapolating the data to man.

The intravenous injection of virulent staphylococci into rabbits in suitable doses causes death. This method has been used more frequently than other virulence tests (Kleiger and Blair, 1940; Chapman et al. 1937). The intradermal injection of rabbits has been used, but different criteria have been accepted as positive findings (Downie, 1937).

Mice are relatively resistant to staphylococci, but they are susceptible to intravenous inoculation (Christie, North, and Parkin, 1946). A 50 per cent mortality rate is obtained in about eight days when four million organisms were inoculated by this route. Characteristic lesions were found in the kidneys and are similar to those produced in rabbits when they are inoculated by the same route. Gorill (1951) compared a small number of coagulase positive strains by this method but there was no difference in virulence among these strains; however, Smith and Dubos (1956) could arrange staphylococci in a continuous series according to their virulence in mice. Another form of virulence test in mice is the

intramuscular injection of 0.2 ml of an eighteen hour broth culture; the size and progress of the lesions serving as the indicator of relative virulence. Comparison with in vitro effects has shown that the size of the swelling obtained, best correlates with the alpha hemolysin production (Selbie and Simon, 1952). Gebhardt et al (1962) showed that coagulase per se failed to exhibit any lethal or virulence activities in suckling mice. The lethal effect was correlated to the production of alpha hemolysin.

The developing chick embryo and its membranes have also been used in virulence testing (Jones, 1946; Knothe, 1952; Wiley, 1961). Frappier and Sonea (1953) found that eighteen to nineteen day old chick embryos are very susceptible to subcutaneous injection of virulent staphylococci.

Many in vitro virulence tests have been developed over the years, but so far none of them give a true indication of virulence. The production of orange pigment, the ability to ferment mannitol and other carbohydrates (Julianelle, 1937) has been associated with virulence, as has the ability to grow in the presence of various dyes (Chapman, Lieb, Berens, and Curcio, 1937).

Of all factors studied in relation to virulence, coagulase (Chapman, Berens, Peters, and Curcio, 1934) and alpha hemolysin production (Christie, North and Parkin, 1946) are regarded as the best indicators

of virulence. Some investigators state that coagulase is the better indicator of virulence while others favor alpha hemolysin production.

It is obvious from the vast amount of literature concerning the virulence of Staph. aureus that no one factor can be implicated as the sole virulence factor. Virulence of Staph. aureus probably depends upon several factors. If the various active substances, such as alpha hemolysin, coagulase, enterotoxins, leucocidin, fibrinolysin, hyaluronidase, etc., that have been described as occurring in filtrates of broth cultures of Staph. aureus are wholly extracellular, then the organism that invades the body does so without the presence of these substances and cannot rely upon them to help initiate an infection. If, however, these substances remain associated with the organism, they are likely to be as important for initiating as for maintaining the organisms from site of entry (Rogers, 1956). The speed at which the various substances are formed or accumulate may also be of importance to the invading organism. A substance, which is formed more rapidly than the defense mechanism can neutralize it, would be most beneficial to the organism in its attack upon a host.

Wiley (1962) has proposed such a virulence mechanism for capsular substance isolated from encapsulated staphylococci. He postulated that the possible role of the capsular substance in the pathogenesis of staphylococcal infection as an aggression, tying up all the host's antibody.

A. Enhancement of Virulence.

Many substances have been credited with the ability to enhance experimental staphylococcal infection. The injection of staphylococci with calcium chloride into rabbits leads to early abscess formation (Rossides, 1927). The intravenous injection of crude dextran of high molecular weight in rabbits is said to enhance the severity of staphylococcal lesions (Sanford, Evans, Preston, 1956).

Nungester, Wolf and Jourdonais (1932) reported that a dose of staphylococci which on intraperitoneal injection failed to cause death in mice would do so if suspended in hog gastric mucin. Starch, agar and gelatin also had some effect, but remarkedly less than mucin.

Since that time the so-called virulence-enhancing effect of mucin has received a great deal of attention. Tunnicliffe (1940) showed that staphylococci suspended in mucin fail to take up a vital stain. She concluded from this that mucin produced a coating and the coating would delay phagocytosis and consequently the organism would multiply, while control saline suspensions of staphylococci are readily phagocytized. These results were further substantiated by Ercoli, Lewis and Harker (1945) who showed that seven hours after intraperitoneal injection of a suspension of staphylococci in saline no visible organisms could be detected, but one-tenth of the same dose in mucin gave confluent growth. In addition they considered that mucin helps in the invasion and spreading from the site of infection.

Elek reported that foreign bodies markedly reduce the minimal dose required to set up lesions in man (Elek, 1956, Elek and Cowen, 1957). On intradermal injection the minimal number of staphylococci required to cause a local formation of pus in man was 1 to 5 million, stitches containing 10,000 organisms on their surface caused severe abscesses in volunteers, and even 100 cocci led to some suppuration. The mechanism whereby the foreign body interferes with the disposal of staphylococci is probably a delay in the defense mechansim whereby the organisms are allowed to multiply.

V. IMMUNITY IN STAPHYLOCOCCAL DISEASE

Since the discovery of staphylococci a great deal of work has been carried out devoted to the immunological aspects of staphylococcal infection; however, very little is still known concerning resistance to such infections.

The vast majority of staphylococcal lesions heal spontaneously, a few lead to invasion of the blood stream and widespread dissemination and death in some cases. Despite the fact that staphylococci can cause death and are so widely spread in nature, man possesses a high degree of natural immunity. What this high degree of resistance is due to is unknown, but it has been inferred that it may be due to the widespread incidence of the organism in the nose. It has been found that many people

are nasal carriers of staphylococci which may or may not be an answer to the high degree of resistance possessed by man.

Panton and Valentine (1929) were able to show by injecting washed suspensions of staphylococci intradermally into rabbits that repeated cutaneous infections gave rise to partial immunity to a large dose. Forssman (1935) found that this type of immunity can be passively transferred to normal animals and that destruction of staphylococci in infected animals was a slow process, even in immunized animals.

Cowan (1939) found that there was some increased resistance to intraveneous infection with heat killed vaccines, but attributed it to non-specific effects. These non-specific results were also observed by other workers (Gallardo and Thompson, 1940).

Vaccines and antitoxins have been used with little success in the treatment and prevention of staphylococcal infections. By 1935 toxoid had given encouraging results in localized staphylococcal infections (Dolman, 1933; Dolman and Kitching, 1935) while antitoxin had apparently aided in recovery in severe cases of acute and generalized infection (Dolman, 1934).

Koenig et al. (1962 a, 1962 b, 1962c) demonstrated antibacterial and antitoxic immunity in experimental staphylococcal infection in mice. The resistance obtained was due to resistance to phagocytosis. Morse (1962) showed that the resistance to phagocytosis was due to distinctive surface

antigens. Animals immunized with heat killed vaccines were readily killed by culture supernatants containing alpha hemolysin, but were protected from infection with viable staphylococci (Koenig et al. 1962c).

Rabbits are known to be more susceptible to staphylococcal infections than mice and they are also very susceptible to the action of staphylococcal alpha hemolysin (Elek, 1959). Boake (1956) found that with preparations of coagulase, rabbits could be actively immunized against lethal intravenous challenge with the homologous strain and that the alpha antitoxin system appeared to play little part in this protection.

Fisher (1957) reported that mice could be immunized with two injections of a toxic supernatant fluid of staphylococcal cultures. The mice were challenged intraperitoneally with a suspension of one hundred million living staphylococci. However, the amount of supernatant fluid required to confer definite immunity, caused scarring, loss of fur and ulceration at the site of subcutaneous injection.

Alpha hemolysin was shown to be responsible for extensive and spreading hemorrhagic necrosis in experimentally infected burns of rabbit skin (Goshi, Cluff, and Johnson, 1961). Acquired resistance was induced by immunization with alpha hemolysin toxoid. Immunity to alpha hemolysin conferred resistance to infection produced by both homologous and heterologous strains of Staph. aureus (Goshi et al. 1961).

Recently Greenberg and Cooper (1960) prepared polyvalent somatic vaccines by lysing staphylococci with deoxyribonuclease. Pooled antigens prepared from selected strains protected hamsters and rabbits against challenge with strains of most types isolated from patients. The route of immunization was of significance in obtaining immunity against certain types of challenge; intradermal immunization was required for protection against challenge by the same route.

Even though there has been some encouraging results in the field of staphylococcal immunity, much remains to be done and the field is still open for investigation.

MATERIALS AND METHODS

I. BACTERIAL STRAINS

Five strains of pathogenic coagulase positive Staph. aureus were selected for use in these studies and will be designated as strain no. 5, 10, 13, 15, 17. Four of the five strains were isolated from humans and one turkey isolate strain (17) was kindly supplied by Dr. Paul B. Carter of Utah State University, Logan, Utah.

All five strains fermented dextrose, lactose, sucrose and mannitol with the production of acid but no detectable gas. Strains 10, 13 and 15 were sensitive to 10 units of penicillin (by plate antibiotic disc method) whereas strains 5 and 17 were resistant to this quantity of antibiotic.

Hemolytic clones of the above strains of Staph. aureus were selected from isolated colonies grown on three per cent rabbit blood agar and seeded in Difco Tryptose phosphate (T. P.) broth.

II. COAGULASE TESTS

Coagulase tests were conducted by using 0.25 ml of broth culture, supernatant or filtrate (undiluted or diluted to 1:1000) to which was added 0.25 ml of undiluted pooled human plasma. This pooled human plasma was a pool of 50 blood samples kindly supplied by the Red Cross.

The results of the use of commercially prepared desiccated rabbit plasma were erratic. The mixtures were incubated at 37° C for 2 hours and readings made at 15, 30, 60 and 120 minutes. They were then placed at 4° C overnight for final test reading. Controls using plasma and saline and plasma and tryptose phosphate broth were included in each test. A positive test was recorded only when a visible coagulum mass was produced. Readings were recorded +, ++ +++, ++++ or negative. A + indicates just preceptible coagulum, while ++++ represents a solid coagulated mass.

III. MOUSE TOXICITY TESTS

Mouse toxicity was determined by the intramuscular injection of 0.1 ml of broth cultures, supernatants or filtrates into 6-14 day old Webster strain white mice. Mice 16 days or older were found to be more resistant to toxin than the 6-14 day old mice. Deaths were recorded hourly for the first 10 hours after inoculation, then two or three times daily for a period of 10 days.

IV. TOXIC PREPARATIONS

Toxic preparations were obtained by culturing selected Staph. aureus strains in tryptose phosphate broth (T. P. broth) and incubating at 37° C for from 24 hours to as long as 20 days with and without added carbon dioxide. The Staph. aureus strains cultured in the presence

of carbon dioxide were seeded to 100 ml of T. P. broth in Erlenmeyer flasks and placed in a desiccator jar. The volume of the desiccator was measured and dry ice added to displace approximately 25 per cent of the air with carbon dioxide. The desiccator jar was then sealed and the cultures were then incubated at 37° C. Duplicate cultures were grown without added carbon dioxide at 37° C.

V. ORIGINAL CULTURES, SUPERNATANTS AND FILTRATES

Supernatants and filtrates were obtained from cultures grown with and without carbon dioxide by centrifuging the original broth cultures at 3500 r. p. m. for two hours at 4° C. Supernatants were filtered either through Millipore, Mandler, or Seitz filters. An International Portable Refrigerated Centrifuge Model PR-2 with head No. 850 a was used.

For convenience, the original cultures, supernatants and filtrates obtained from these cultures will be designated as:

(1) Cultures grown in the presence of carbon dioxide--25% CO_2 culture, 25% CO_2 supernatant, and 25% CO_2 filtrate.

(2) Cultures grown without added carbon dioxide--culture no CO_2, supernatant no CO_2, and filtrate no CO_2.

VI. IMMUNIZATION OF RABBITS

Two rabbits were immunized with a Seitz filtrate of Staph. aureus strain 15 tryptose phosphate broth culture grown under atmospheric

conditions and incubated for 9 days at 37° C. Two other rabbits were immunized with a Seitz filtrate of a Staph. aureus strain #15 tryptose phosphate broth culture grown in the presence of 25% carbon dioxide incubated for 9 days at 37° C. Two more rabbits were immunized with the preceeding Seitz filtrate which had been placed in boiling water for ten minutes. All rabbits received 5 intramuscular injections given at 5 day intervals.

The following immunization schedule was followed:

Amount injected	Dilution
0.1 ml	1:5
0.2 ml	1:5
0.1 ml	undiluted
0.2 ml	undiluted
0.5 ml	undiluted

VII. GLASSWARE PREPARATION

All glassware used was placed in distilled water with Hemo-sol, boiled for 1 hour and allowed to cool at room temperature. The glassware was then rinsed in tap water 4 times and in distilled water 4 times. It was placed in 1 per cent hydrochloric acid solution overnight, rinsed in tap water 4 times, in distilled water 4 times and allowed to air dry. All glassware was autoclaved prior to use.

RESULTS

I. STUDIES TO DETERMINE THE EFFECT OF STAPHYLOCOCCAL CULTURE SUPERNATANTS UPON MICE OF DIFFERENT AGE GROUPS

Adult mice are notably resistant to experimental staphylococcal infections (Christie et al., 1946); however, they possess the advantage of being the most economical and convenient laboratory animal to use in studying staphylococcal infections. With this in mind, suckling mice in the age range of 6-18 days were injected with the supernatants obtained from staphylococcal cultures to determine mouse susceptibility. These results are presented in Tables 1 and 2.

II. THE EFFECT OF PROLONGED INCUBATION UPON THE COAGULASE TITER AND MOUSE TOXICITY

Two strains of Staph. aureus, #13 and #15, were seeded to tryptose phosphate broth and incubated at 37° C for a total incubation period of twenty days. Coagulase tests were carried out on aliquots of cultures following incubation periods of 1, 2, 3, 4, 5, 10, 15 and 20 days.

Mouse toxicity tests were carried out by injection of 0.1 ml of the culture intramuscularly into 8-12 day old suckling mice. The results are presented in Table 3.

TABLE 1

Effect of injecting mice of different age groups with supernatant obtained from Staph. aureus strain #15 culture incubated for 3 days at 37° C

Culture	No. Mice Injected	Amount Injected	Age of Mice in days	Results (deaths in hrs.)
Supernatant obtained from Staph. aureus culture incubated for 3 days at 37° C	6	0.1 ml I.M.	6	6/6* 3 dead 3 hrs. 2 dead 3.5 hrs. 1 dead 10.5 hrs.
	8	"	9	8/8 1 dead 3.5 hrs. 5 dead 6.5 hrs. 2 dead 8 hrs.
	10	"	11	9/10 4 dead 3 hrs. 2 dead 3.5 hrs. 3 dead 4.5 hrs.
	10	"	14	10/10 1 dead 4 hrs. 1 dead 4.5 hrs. 6 dead 6.5 hrs. 1 dead 10.5 hrs. 1 dead 12.5 hrs.
	9	"	16	9/9 1 dead 5 hrs. 4 dead 6.5 hrs. 1 dead 8 hrs. 2 dead 11 hrs. 1 dead 11.5 hrs.
	6	"	18	0/10**

* 6/6: 6 died out of 6 injected.

** No deaths 10 days after injection.

TABLE 2

Effect of injecting mice of different age groups with supernatant obtained from Staph. aureus strain #15 culture incubated for 7 days at 37° C without added CO_2

Culture	No. mice Injected	Amount Injected	Age of Mice in Days	Results (death in hrs.)
Supernatant obtained from Staph. aureus culture incubated for 7 days at 37° C	6	0.1 ml I.M.	9	6/6* 4 dead 2.5 hrs. 2 dead 3 hrs.
	8	"	12	8/8 2 dead 3 hrs. 4 dead 3.5 hrs. 2 dead 4.5 hrs.
	9	"	14	9/9 8 dead 3.5 hrs. 1 dead 4 hrs.
	7	"	17	7/7 3 dead 9 hrs. 4 dead 20 hrs.
	6	"	18	1/6** 1 dead 24 hrs.

* 6/6: 6 died out of 6 injected.
** No further deaths 10 days after injection.

TABLE 3

Effect of incubation time upon coagulase titer and mouse lethality of broth cultures of Staph. aureus strains #13 and #15 without added CO_2

Culture		Incubation Time in Days at 37° C							
		1	2	3	4	5	10	15	20
Staph. aureus strain #13	Coagulase Titer	+++1:50	+++1:100	+1:200	+1:200	+++1:100	+1:100	+1:200	+++1:200
	Lethality	0/4	4/4	3/4	4/4	4/4	4/4	2/4	4/4
Staph. aureus strain #15	Coagulase Titer	1:200	+++1:100	+1:200	+1:200	+1:200	++1:100	++1:100	+1:100
	Lethality	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4

III. THE EFFECTS OF FREEZING BROTH CULTURES OF STAPH. AUREUS STRAIN #13 AND #15

A. Effect of freezing and thawing on coagulase titer and mouse lethality of a broth culture of strain #15.

Duthie (1954) reported the existence of two different kinds of coagulase; one a soluble coagulase which is liberated into the medium and the other a bound coagulase. To determine if broth cultures of staphylococci could be frozen and thawed without any appreciable increase in coagulase titer as a result of the release of the bound coagulase, or a decrease in coagulase titer or toxic activity, a broth culture of Staph. aureus strain #15 was incubated for 6 days at 37° C without added CO_2 and frozen and thawed several times for a period of four days. Coagulase titer and mouse toxicity were determined. The results are presented in Table 4.

B. Effect of prolonged freezing.

Since freezing and thawing of a broth culture did not affect the coagulase or toxic activity, the effect of prolonged freezing was studied.

Broth cultures of Staph. aureus strain #13 and #15 were incubated at 37° C without increased CO_2 for 1, 2, 3, 4, and 5 days. Aliquots of these cultures were then frozen for seven months (-26° C), then re-tested for coagulase activity and mouse toxicity. See Tables 5-9 for the results of this experiment.

TABLE 4

Coagulase titer and mouse lethality of a broth culture of Staph. aureus strain #15 after being frozen and thawed several times.

Culture	Coagulase test read after indicated time in minutes	Dilutions					
		undil.	1:10	1:50	1:100	1:200	1:1000
Staph. #15	15	-	-	-	-	-	-
incubated	30	-	-	-	-	-	-
for 6 days	60	+	-	-	-	-	-
at 37° C	120	++++	+++	++	-	-	-
	overnight 4° C	++++	++++	++++	+++	+	-

5 mice died of 5 inoculated with the undiluted culture within 24 hours after injection.

Coagulase titer before freezing and thawing was +++1:200.

TABLE 5

Coagulase titer of broth culture of Staph. aureus strain #13 after 1, 2, 3, 4, and 5 days' incubation at 37° C

Culture	Coagulase test read after indicated time in minutes	Days Incubated at 37° C				
		1	2	3	4	5
Staph. aureus strain #13	15	-	+u*	+u	+u	++u
	30	+++u	++u	++u	+++u	++u
	60	+++1:10	+++1:10	+++1:10	++1:10	++1:10
	120	++1:100	+1:100	+++1:100	+++1:50	+1:50
	overnight 4° C	+++1:200	+++1:200	+++1:200	++1:200	+1:200
Controls		-	-	-	-	-

u* = undiluted

TABLE 6

Coagulase titers of broth cultures of Staph. aureus strain #13 after 1, 2, 3, 4, and 5 days' incubation at 37° C then placed at -26° C for a period of seven months.

Culture	Coagulase test read after indicated time in minutes	Days Incubated at 37° C				
		1	2	3	4	5
Staph. aureus strain #13 frozen for seven months	15	-	-	-	-	-
	30	+u*	++u	++u	++u	++u
	60	++u	++u	+++u	+++u	+++u
	120	++1:10	+++u	+++1:10	+++u	++1:10
	overnight 4° C	+1:100	+1:50	+1:200	+1:50	+1:200
Controls		-	-	-	-	-

u* = undiluted

TABLE 7

Coagulase titers of broth cultures of Staph. aureus strain #15 after 1, 2, 3, 4, and 5 days' incubation at 37° C

Culture	Coagulase test read after indicated time in minutes	Days Incubated at 37° C				
		1	2	3	4	5
Staph. aureus strain #15	15	-	-	-	-	-
	30	+++u	++u	++u	+++u	++u
	60	+++1:10	+++1:10	+++1:10	++1:10	++1:10
	120	++1:10	+1:100	++1:100	++1:100	++1:50
	overnight 4° C	++1:200	++1:200	++1:200	++1:200	+1:100
Controls		-	-	-	-	-

u* = undiluted

TABLE 8

Coagulase titers of broth cultures of Staph. aureus strain #15 after 1, 2, 3, 4, and 5 days' incubation at 37° C then placed at -26° C for a period of seven months.

Culture	Coagulase test read after indicated time in minutes	Days Incubated at 37° C				
		1	2	3	4	5
Staph. aureus strain #15 frozen for seven months	15	-	-	-	-	-
	30	-	-	-	-	-
	60	+u*	+u	+u	++u	++u
	120	++u	+1:10	++u	++1:10	++1:10
	overnight 4° C	+++1:10	+1:50	++1:10	+1:100	+1:50
Controls		-	-	-	-	-

u* = undiluted

TABLE 9

Results of mouse inoculations with cultures of Staph. #13 and Staph. #15 incubated for 72 and 96 hours at 37° C then placed at -26° C for a period of seven months.

Culture	No. mice injected	Amount injected	Results No. deaths	Coagulase titer	Coagulase titer before freezing
Staph. aureus strain #13 72 hour broth culture	4	0.1 ml	3/4	+1:200	+++1:200
Staph. aureus strain #13 96 hour broth culture	4	0.1 ml	0/4	+1:50	++1:200
Staph. aureus strain #15 72 hour broth culture	4	0.1 ml	4/4	+1:100	++1:200
Staph. aureus strain #15 96 hour broth culture	4	0.1 ml	4/4	+1:100	++1:200

IV. FILTRATION OF SUPERNATANT OF A 72 HOUR BROTH CULTURE OF STAPH. AUREUS STRAIN #15

In this experiment effects of filtration were studied to determine if either the coagulase or toxic activity of staphylococcal cultures would be affected. A 72 hour broth culture of Staph. aureus strain #15 incubated at 37° C without added CO_2 was centrifuged for two hours at 3500 r.p.m. and 4° C. Aliquots of the supernatant fluid were filtered through either a Mandler, Seitz, or Millipore filter.

Mice were injected with each of the filtrates and coagulase titers were determined. Coagulase titers are presented in Table 10 and Table 11 shows the results of the mouse inoculations.

V. THE EFFECT OF TEMPERATURE AND SEITZ FILTRATION UPON COAGULASE AND LETHALITY OF A BROTH CULTURE OF STAPH. AUREUS STRAIN #15

In this experiment, Staph. aureus strain #15 was grown in tryptose phosphate broth for 72 hours to 7 days at 37° C. Aliquots of the broth cultures were placed in boiling water for 10 minutes and in a 56° C water bath for 30 minutes. The supernatants and Seitz filtrates obtained from the cultures were tested for coagulase and lethal activity; however, they were not heat treated.

TABLE 10

Coagulase titer of a 72 hour broth culture and filtrates of Staph. aureus strain #15.

Culture	Coagulase test read after indicated time in minutes	Dilutions					
		und	1:10	1:50	1:100	1:200	1:1000
Original culture 72 hour 37° C	15	-	-	-	-	-	-
	30	-	-	-	-	-	-
	60	+++	+	-	-	-	-
	120	++++	++++	+++	+	-	-
	overnight	++++	++++	++++	++++	+++	-
Super-natant	15	-	-	-	-	-	-
	30	-	-	-	-	-	-
	60	++	-	-	-	-	-
	120	++++	++++	-	-	-	-
	overnight	++++	++++	++++	++	±	-
Mandler filtrate	15	-	-	-	-	-	-
	30	-	-	-	-	-	-
	60	-	-	-	-	-	-
	120	-	-	-	-	-	-
	overnight	±	-	-	-	-	-
Millipore filtrate	15	-	-	-	-	-	-
	30	-	-	-	-	-	-
	60	++	-	-	-	-	-
	120	++++	++++	+	-	-	-
	overnight	++++	++++	++++	+++	+	-
Seitz filtrate	15	-	-	-	-	-	-
	30	-	-	-	-	-	-
	60	-	-	-	-	-	-
	120	-	-	-	-	-	-
	overnight	-	-	-	-	-	-

TABLE 11

Results of animal inoculations with original culture, supernatant, and filtrates of Staph. aureus strain #15.

Culture	No. mice	Amount injected	Results No. deaths	Coagulase titer
Original culture	4	0.1 ml I.M.	4/4*	+++1:200
Supernatant	4	"	4/4*	++1:100
Millipore filtrate	4	"	4/4*	+1:200
Seitz filtrate	5	"	4/5*	---
Mandler filtrate	5	"	5/5*	± undiluted

* died within 4 hours after injection.

Aliquots of the original culture incubated for 72 hours at 37° C were placed in boiling water for various periods of time and tested for coagulase and lethal activity. Results are presented in Tables 12, 13, 14, and 15.

VI. EFFECT OF TEMPERATURE ON COAGULASE AND TOXIC ACTIVITY OF DIFFERENT STRAINS OF STAPH. AUREUS

A. The effect of temperature on coagulase and toxic activity of the original culture, supernatant and Seitz filtrate of a 9-day culture of Staph. aureus strain #15.

Staphylococcal alpha hemolysin has been reported to be highly thermostable. Tager (1941) obtained active toxic preparations after boiling staphylococcal supernatants for 15 minutes. It has been reported that coagulase is relatively heat resistant and is capable of withstanding 60° C for thirty minutes (Smith and Hale, 1944). In the following experiments the effect of temperature was studied.

In the first experiment Staph. aureus strain #15 was seeded to 100 ml of tryptose phosphate broth and allowed to incubate at 37° C with added CO_2 for a period of 9 days.

After this incubation period, aliquots of the original culture, supernatant and Seitz filtrate were placed in boiling water for various periods of time. These heat treated preparations were then tested

TABLE 12

Coagulase titers and lethality for mice of Staph. aureus cultures of strain #15 under varying conditions.

Staph. aureus culture tryptose phosphate broth	Coagulase titer	Lethality for 6-10 day old mice
72 hr., 37° C	+1:200	10/10 1 dead 8 hours 9 dead 9.5 hours
7 days 37° C	++1:200	10/10 10 dead 4 hours
72 hrs., 37° C placed in boiling water for 10 min.	trace- undiluted	0/10
7 days, 37° C placed in boiling water for 10 min.	negative	0/10
72 hrs., 37° C placed in 56° C water bath 30 min.	negative	0/10
7 days, 37° C placed in 56° C water bath 30 min.	negative	0/10
72 hrs., 37° C centrifuged 2 hrs. 3500 r.p.m. supernatant tested	+++1:10	10/10 1 dead 24 hours 9 dead 4 days
7 days, 37° C centrifuged 2 hrs. 3500 r.p.m. supernatant tested	negative	10/10 6 dead 6 hours 3 dead 9 hours 1 dead 11 hours
72 hrs., 37° C Seitz-filtered	negative	0/10
7 days, 37° C Seitz-filtered	negative	8/10 3 dead 6.5 hours 5 dead 8 hours

TABLE 13

Effect of time of boiling temperature on a 72 hr. broth culture of Staph. aureus strain #15.

Culture	No. minutes in boiling water	Coagulase titer	Lethality for 6-10 day old mice
Original culture	0	+1:100	1/10 1 dead 24 hrs.
	3	++undiluted	0/10
	6	++undiluted	0/10
	9	++undiluted	0/10
	12	++undiluted	0/10
	15	++undiluted	0/10

TABLE 14

Effect of time of boiling temperature on a 7 day broth culture of Staph. aureus strain #15.

Culture	No. minutes in boiling water	Coagulase titer	Lethality for 6-10 day old mice
Original culture	0	+1:200	1/10 10 dead 5 hrs.
	3	+undiluted	0/10
	6	+undiluted	0/10
	9	+undiluted	0/10
	12	negative	0/10
	15	negative	0/10

TABLE 15

Effect of time of boiling temperature on the supernatant of a 7 day broth culture of Staph. aureus strain #15.

Culture	No. minutes in boiling water	Coagulase titer	Lethality for 6-10 day old mice
Supernatant obtained from a 7 day broth culture	0	+undiluted	8/8* 6 dead 3 hrs. 1 dead 4 hrs. 1 dead 5.5 hrs.
	3	negative	0/8
	6	negative	0/8
	9	negative	0/8
	12	negative	0/8
	15	negative	0/8

* 8 dead of 8 inoculated.

for coagulase and toxic activity. Toxicity was determined by intramuscular injection of 0.1 ml of the preparation being tested into suckling mice. Tables 16, 17 and 18 show the results of this experiment.

B. Effect of temperature on coagulase and toxic activity of 5 strains of Staph. aureus.

In view of the results obtained in the previous experiment, that is, that even after 15 minutes at 96° C the original cultures, supernatants and Seitz filtrates were still toxic for suckling mice, it was decided to test the effect of temperature on coagulase and toxic activity on cultures of different strains of Staph. aureus. Two flasks of tryptose phosphate broth were inoculated with the organisms and allowed to incubate at 37° C for a period of 9 days. One set of flasks was incubated with 25% CO_2 and the other set was incubated without the presence of added CO_2. After this incubation period, 10 ml aliquots of the original culture and Seitz filtered preparations of these cultures were placed in boiling water (96° C*) for a period of 0, 5, 10 and 15 minutes. These heat treated preparations of the original culture and Seitz filtrate were then tested for the presence of coagulase and toxic activity in suckling mice.

* Water boils at 96° C at the altitude of the laboratory, about 4300'.

TABLE 16

Effect of time of boiling temperature on the original culture of Staph. aureus strain #15 incubated 9 days at 37° C and 25% CO_2.

Culture	No. minutes placed in boiling water	Lethality for 6-10 day old mice	Coagulase titer
Original 9 day	0	10/10* 1 dead 1 hr. 5 dead 1 3/4 hrs. 4 dead 2 1/4 hrs.	+1:50
	3	10/10 4 dead 3 hrs. 5 dead 3 1/2 hrs. 1 dead 6 hrs.	negative
	6	10/10 2 dead 3 hrs. 3 dead 3 1/2 hrs. 1 dead 4 hrs. 3 dead 6 hrs.	negative
	9	0/10	negative
	12	4/10 3 dead 10 hrs. 1 dead 26 hrs.	negative
	15	5/10 1 dead 8 hrs. 1 dead 10 hrs. 1 dead 12 hrs. 2 dead 48 hrs.	negative

* 10 mice died of 10 inoculated.

TABLE 17

Effect of time of boiling temperature on the supernatant of Staph. aureus strain #15 culture incubated 9 days at 37° C and 25% CO_2.

Culture	No. minutes placed in boiling water	Lethality for 6-10 day old mice	Coagulase titer
Supernatant	0	10/10* 3 dead 1 hr. 3 dead 1 3/4 hr. 3 dead 2 1/4 hr. 1 dead 2 3/4 hrs.	negative
	3	10/10 3 dead 3 hrs. 7 dead 3 1/2 hrs.	negative
	6	9/9 4 dead 3 hrs. 5 dead 3 1/2 hrs.	negative
	9	10/10 4 dead 4 1/2 hrs. 3 dead 5 1/2 hrs. 2 dead 7 hrs. 1 dead 8 hrs.	negative
	12	10/10 10 dead 5 hrs.	negative
	15	10/10 1 dead 4 1/2 hrs. 3 dead 5 hrs. 2 dead 7 hrs. 1 dead 8 hrs. 1 dead 10 hrs. 1 dead 72 hrs. 1 dead 4 days	negative

* 10 mice died of 10 inoculated.

TABLE 18

Effect of time of exposure to boiling temperature on Seitz filtrates of Staph. aureus strain #15 culture incubated 9 days at 37° C and 25% CO_2.

Culture	No. minutes placed in boiling water	Lethality for 6-10 day old mice	Coagulase titer
Seitz filtrate	0	7/7* 6 dead 1 hr. 1 dead 2 hrs.	negative
	3	10/10 1 dead 2 1/2 hrs. 7 dead 3 hrs. 2 dead 4 hrs.	negative
	6	10/10 1 dead 2 1/2 hrs. 2 dead 3 hrs. 7 dead 4 hrs.	negative
	9	10/10 1 dead 3 hrs. 4 dead 4 hrs. 4 dead 5 hrs. 1 dead 7 hrs.	negative
	12	10/10 1 dead 2 1/2 hrs. 9 dead 3 hrs.	negative
	15	9/9 5 dead 5 hrs. 3 dead 7 hrs. 1 dead 9 hrs.	negative

* seven mice died of seven inoculated.

Coagulase tests were carried out by employing 0.25 ml of the diluted treated samples to which was added 0.25 ml of pooled human plasma. The mixtures were incubated at 37° C for two hours, readings were made at 15, 30, 60 and 120 minutes. The mixtures were then placed at 4° C overnight before final reading.

Mouse toxicity tests were carried out by the intramuscular injection of 0.1 ml of the heat treated samples into ten 6-10 day old Webster strain white mice. Deaths were recorded hourly for the first twelve hours after inoculation. (Animals were observed for a period of ten days).

The Seitz filtrates were prepared by centrifuging the original broth culture for 30 minutes at 3500 r.p.m. and 4° C to remove the bulk of organisms. The supernatant fluid was then filtered through a Seitz filter. Tables 19-28 show the effects of time of exposure to boiling temperature on coagulase and toxic activity of cultures and filtrates of different strains of Staph. aureus.

VII. EXPERIMENT TO DETERMINE WHETHER THE HEAT LABILE AND THE HEAT STABLE FRACTIONS OF STAPH. AUREUS TOXIN ARE ANTIGENICALLY DIFFERENT

Tager (1941) suggested the presence of two fractions of alpha toxin. Heating at 56° to 60° C for thirty minutes inactivated both

TABLE 19

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #5 original culture grown without added CO_2 and Seitz filtrate prepared from the culture.

Preparation	No. minutes placed in boiling water	Mortality	Coagulase titer
Original culture 9 days 37° C no CO_2	0	10/10* 10 dead 24 hrs.	+1:50
	5	10/10 3 dead 5 days 7 dead 11 days	negative
	10	10/10 10 dead 11 days	negative
	15	10/10 10 dead 12 days	negative
9 day Seitz filtrate no CO_2	0	0/10	negative
	5	0/10	negative
	10	0/10	negative
	15	0/10	negative

* 10 animals died of 10 inoculated.

TABLE 20

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #10 original culture grown without added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
Original culture 9 day 37° C no CO_2	0	10/10*	10 dead 24 hrs.	+1:50
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative
9 day Seitz filtrate no CO_2	0	0/10		negative
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative

* 10 animals died of 10 inoculated.

TABLE 21

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #13 original culture grown without added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality	Coagulase titer
9 day original culture no CO_2	0	0/10*	++1:100
	5	0/10	+1:10
	10	0/10	+1:10
	15	0/10	+1:10
9 day Seitz filtrate no CO_2	0	0/10	negative
	5	0/10	negative
	10	0/10	negative
	15	0/10	negative

* 0 animals died of 10 inoculated.

TABLE 22

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #15 original culture grown without added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
Original culture 9 days no CO_2	0	9/10*	3 dead 3.5 hrs. 3 dead 4.75 hrs. 1 dead 5 hrs. 2 dead 6 hrs.	+++1:10
	5	0/10		+ undiluted
	10	0/10		+ undiluted
	15	0/10		+ undiluted
9 day Seitz filtrate no CO_2	0	10/10	9 dead 3.75 hrs. 1 dead 4 hrs.	negative
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative

* 9 animals died of 10 inoculated.

TABLE 23

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #17 original culture grown without added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
Original culture 9 days 37° C no CO_2	0	10/10*	10 dead 2.5 hrs.	+1:10
	5	0/10		++undiluted
	10	0/10		++undiluted
	15	0/10		++undiluted
9 day Seitz filtrate no CO_2	0	0/10		negative
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative

* 10 animals died of 10 inoculated.

TABLE 24

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #5 original culture grown in the presence of added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
Original culture 9 days 37° C +CO_2	0	10/10*	1 dead 1.5 hrs. 9 dead 2.5 hrs.	++1:10
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative
9 day culture with CO_2 then Seitz filtered	0	10/10	2 dead 1.5 hrs. 8 dead 2.5 hrs.	negative
	5	10/10	3 dead 4.5 hrs. 7 dead 7.5 hrs.	negative
	10	6/10	3 dead 9 hrs. 3 dead 12 hrs.	negative
	15	0/10		negative

* 10 animals died of 10 inoculated.

TABLE 25

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #10 original culture grown in the presence of added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
Original culture	0	4/10*	4 dead 24 hrs.	negative
9 day 37° C +CO_2	5	0/10		negative
	10	4/8	4 dead 72 hrs.	negative
	15	0/8		negative
9 day culture with CO_2 then Seitz filtered	0	0/10		negative
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative

* 4 animals died of 10 inoculated.

TABLE 26

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #13 original culture grown in the presence of added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
9 day original culture +CO_2	0	10/10*	10 dead 24 hrs.	++1:10
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative
9 day culture with CO_2 then Seitz filtered	0	9/10	3 dead 6 hrs. 3 dead 7 hrs. 3 dead 8 hrs.	negative
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative

* 10 animals died of 10 inoculated.

TABLE 27

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #15 original culture grown in the presence of added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
Original 9 day culture $+CO_2$	0	10/10*	6 dead 3 hrs. 4 dead 4 hrs.	+undiluted
	5	7/10	1 dead 6 hrs. 1 dead 10 hrs. 1 dead 24 hrs. 4 dead 6 days	negative
	10	9/10	4 dead 8 hrs. 2 dead 10 hrs. 1 dead 24 hrs. 1 dead 48 hrs. 1 dead 6 days	negative
	15	3/10	1 dead 12 hrs. 2 dead 6 days	negative
9 day culture with CO_2 then Seitz filtered	0	10/10	7 dead 1.75 hrs. 3 dead 2.5 hrs.	negative
	5	7/10	5 dead 7 hrs. 2 dead 8.5 hrs.	negative
	10	10/10	6 dead 4 hrs. 2 dead 4.5 hrs. 2 dead 5 hrs.	negative
	15	8/10	3 dead 5 hrs. 3 dead 7 hrs. 2 dead 8.5 hrs.	negative

* 10 animals died of 10 inoculated.

TABLE 28

Effect of time of exposure to boiling temperature on coagulase and toxic activity of Staph. aureus strain #17 original culture grown in the presence of added CO_2 and Seitz filtrate prepared from this culture.

Preparation	No. minutes placed in boiling water	Mortality		Coagulase titer
Original culture 9 day 37° C +CO_2	0	10/10*	10 dead 2.5 hrs.	+undiluted
	5	0/10		negative
	10	0/10		negative
	15	0/10		negative
9 day culture with CO_2 then Seitz filtered	0	10/10	2 dead 1.75 hrs. 8 dead 2.5 hrs.	negative
	5	10/10	7 dead 3 hrs. 1 dead 3.5 hrs. 2 dead 4.0 hrs.	negative
	10	10/10	6 dead 3.0 hrs. 1 dead 4.0 hrs. 3 dead 4.5 hrs.	negative
	15	10/10	4 dead 4.0 hrs. 3 dead 4.5 hrs. 1 dead 5.0 hrs. 6 dead 6.0 hrs.	negative

* 10 animals died of 10 inoculated.

fractions, whereas one-half of the activity was restored upon boiling for ten minutes; therefore, he suggested a heat labile and a heat stable fraction. The following experiment was carried out to determine if these two fractions are antigenically different.

Two rabbits were immunized with each of the following preparations: Two flasks of tryptose phosphate broth were inoculated with Staph. aureus strain #15 and allowed to incubate at 37° C for a period of 9 days. One flask was incubated under 25% CO_2 and the other without the presence of CO_2. After this incubation period, Seitz filtrates were prepared by centrifuging the original broth culture for 30 minutes at 3500 r.p.m. and 4° C to remove the bulk of organisms. The supernatant fluid was then filtered through a Seitz filter. A 10 ml aliquot of the Seitz filtrate obtained from the culture grown in the presence of 25% CO_2 was placed in boiling water (96° C) for a period of 10 minutes. Two rabbits were immunized with this preparation. Two more were immunized with the nonheated Seitz filtrate (25% CO_2) and two more were immunized with the Seitz filtrate obtained from the culture grown in the absence of CO_2. The immunization schedule followed was as previously described in the materials and methods section.

One week after the last injection, the rabbits were bled and the antisera tested. Each filtrate was diluted 1:2, 1:8, 1:16 and mixed in an equal volume with each antisera and incubated for 1 hour at 37° C.

Mouse toxicity tests were carried out by the intramuscular injection of 0.1 ml of each of the samples into four 6-10 day old Webster strain white mice. These results will be found in Tables 29 and 30.

Four mice were also injected with each filtrate diluted 1:2 and 1:4 to determine the effects of dilution on the toxic activity of these preparations. See Table 31 for these results.

The antiserums will be referred to as antiserum #1, 2, 3, 4, and 5.

Antiserums 1 and 2 were prepared from rabbits immunized with no CO_2 cultures then Seitz filtered.

Antiserums 3 and 4 were prepared from rabbits immunized with 25% CO_2 cultures then Seitz filtered (nonheated).

Antiserum 5 was prepared from rabbits immunized with 25% CO_2 cultures then Seitz filtered (heated 10 minutes in boiling water).

VIII. MOUSE PROTECTION STUDIES

This experiment was carried out to determine if mice could be protected with antiserum from rabbits immunized with Seitz filtrates described in the previous experiment. The same procedure was followed with the exception that mice were injected with a mixture of each antiserum and homologous and heterologous antigen.

No significant protection was noted as is evident from Tables 32-35.

TABLE 29

Results of mouse toxicity tests employing antiserum + Seitz filtrate of culture (no CO_2)

Preparation	Dilution of filtrate	Mortality		Coagulase titer
Antiserum #1 + no CO_2 filtrate	undil.	3/4	3 dead 24 hrs.	negative
	1:2	0/4		negative
	1:4	0/4		negative
	1:8	0/4		negative
	1:16	0/4		negative
Antiserum #2 + no CO_2 filtrate	undil.	0/4		negative
	1:2	0/4		negative
	1:4	0/4		negative
	1:8	0/4		negative
	1:16	0/4		negative

TABLE 30

Results of mouse toxicity tests employing antiserum + Seitz filtrate (25% CO_2)

Preparation	Dilution of filtrate	Mortality		Coagulase titer
Antiserum #3 + 25% CO_2 filtrate	undil.	4/4	4 dead 2 hrs.	negative
	1:2	4/4	4 dead 2 hrs.	"
	1:4	0/4		"
	1:8	0/4		"
	1:16	0/4		"
Antiserum #4 + 25% CO_2 filtrate	undil.	4/4	4 dead 2.5 hrs.	negative
	1:2	4/4	4 dead 3 hrs.	"
	1:4	0/4		"
	1:8	0/4		"
	1:16	0/4		"
Antiserum #5 + boiled 25% CO_2 filtrate (10 minutes)	undil.	0/4		negative
	1:2	0/4		"
	1:4	0/4		"
	1:8	0/4		"
	1:16	0/4		"

TABLE 31

Results of mouse toxicity tests employing Seitz filtrates obtained from cultures grown with and without added CO_2 and filtrates plus normal serum.

Preparation	Dilution of filtrate	Mortality	
9 day no CO_2	undil.	4/4*	4 dead 10 hrs.
	1:2	4/4	4 dead 12 hrs.
	1:4	0/4	
9 day 25% CO_2	undil.	4/4	4 dead 4 hrs.
	1:2	4/4	4 dead 5 hrs.
	1:4	0/4	
9 day boiled 10 minutes	undil.	2/4	2 dead 8 hrs.
	1:2	0/4	
	1:4	0/4	
9 day no CO_2 filtrate + normal serum	undil.	3/4	3 dead 10 hrs.
9 day 25% CO_2 filtrate + normal serum	undil.	4/4	4 dead 6 hrs.
9 day 25% CO_2 filtrate boiled 10 minutes + normal serum	undil.	1/4	1 dead 12 hrs.

* 4 animals died of 4 inoculated.

TABLE 32

Results of inoculating suckling mice with a mixture of antiserum #1 and homologous and heterologous antigen serum constant 1:8 dilution but filtrate diluted.

Preparation	Dilution of filtrate		Mortality	Coagulase titer
Antiserum #1 + 9 day no CO_2 filtrate	undil.	3/4*	3 dead 24 hrs.	negative
	1:2	0/4		"
	1:4	0/4		"
	1:8	0/4		"
Antiserum #1 + 9 day 25% CO_2 filtrate	undil.	4/4	3 dead 2 3/4 hrs. 1 dead 3 1/4 hrs.	negative
	1:2	4/4	3 dead 2 3/4 hrs. 1 dead 4 hrs.	"
	1:4	2/4	1 dead 6 hrs. 1 dead 24 hrs.	"
	1:8	0/4		"
Antiserum #1 + 9 day 25% CO_2 filtrate boiled 10 min.	undil.	0/4		negative
	1:2	0/4		"
	1:4	0/4		"
	1:8	0/4		"

* 3 animals died of 4 inoculated.

TABLE 33

Results of inoculating suckling mice with Antiserum #3 and homologous and heterologous antigen serum constant 1:8 dilution but filtrate diluted.

Preparation	Dilution of filtrate	Mortality		Coagulase titer
Antiserum #3 + 9 day no CO_2 filtrate	undil.	0/4*		negative
	1:2	0/4		"
	1:4	0/4		"
	1:8	0/4		"
Antiserum #3 + 9 day 25% CO_2 filtrate	undil.	4/4	4 dead 2 hrs.	negative
	1:2	4/4	4 dead 2 hrs.	"
	1:4	0/4		"
	1:8	0/4		"
Antiserum #3 + 9 day 25% CO_2 filtrate boiled 10 min.	undil.	0/4		negative
	1:2	0/4		"
	1:4	0/4		"
	1:8	0/4		"

* 0 animals died of 4 inoculated.

TABLE 34

Results of inoculating suckling mice with a mixture of antiserum 5 and homologous and heterologous antigen serum constant 1:8 dilution but filtrate diluted.

Preparation	Dilution of filtrate	Mortality		Coagulase titer
Antiserum #5 + 9 day no CO_2 filtrate	undil.	0/4*		negative
	1:2	0/4		"
	1:4	0/4		"
	1:8	0/4		"
Antiserum #5 + 9 day 25% CO_2 filtrate	undil.	4/4	1 dead 3 1/4 hrs. 3 dead 4 1/2 hrs.	negative
	1:2	4/4	4 dead 5 hrs.	"
	1:4	4/4	1 dead 5 1/2 hrs. 3 dead 24 hrs.	"
	1:8	0/4		"
Antiserum #5 + 9 day 25% CO_2 filtrate boiled 10 min.	undil.	0/4		negative
	1:2	0/4		"
	1:4	0/4		"
	1:8	0/4		"

* 0 animals died of 4 inoculated.

TABLE 35

Results of inoculating suckling mice with Seitz filtrates obtained from broth cultures grown under various conditions.

Preparation	No. mice inoculated	Amount injected	Mortality	Coagulase titer
9 day Seitz filtrate no CO_2	10	0.1 ml I.M.	10/10* 9 dead 3 3/4 hrs. 1 dead 4 hrs.	negative
9 day Seitz filtrate + 25% CO_2	10	0.1 ml I.M.	10/10 7 dead 1 3/4 hrs. 3 dead 2 1/4 hrs.	negative
9 day Seitz filtrate +25% CO_2 placed in boiling water 10 min.	10	0.1 ml I.M.	8/10 6 dead 4 hrs. 2 dead 4 1/2 hrs.	negative

* 10 animals died of 10 inoculated.

IX. EXPERIMENT TO DETERMINE WHETHER OR NOT COAGULASE IS PRODUCED IN VIVO

The production of coagulase has been customarily taken as an in vitro reaction indicating potential pathogenicity of Staph. aureus. Ekstedt and Yotis (1960) postulated that coagulase protects the microorganisms from the natural defense mechanisms of the host. Smith et al. (1947) stated that coagulase is essential for the organisms to gain a foothold in the tissues. However, there is no evidence as to whether or not coagulase is produced in vivo. In this experiment an attempt was made to determine if coagulase is produced in vivo. Staph. aureus strain #15 was seeded to tryptose phosphate broth and allowed to incubate at 37° C for a period of three days. The culture was then centrifuged to remove the bulk of the organisms. The organisms were then washed in saline and 0.2 ml of packed cells were suspended in saline so as to have a final volume of 6 ml; 0.5 ml aliquots were then placed in a 56° C water bath for various periods of time ranging from 35-75 minutes. Each aliquot was tested for viability and coagulase activity. Heating for 75 minutes at 56° C did not destroy the viability of this organism nor its ability to produce coagulase as is noted in Table 36.

A negative coagulase test was obtained with the 75 minute heat treated sample, so suckling mice (14-18 days old) were inoculated

TABLE 36

Results of coagulase tests on broth cultures inoculated with heat treated and nonheat treated Staph. aureus strain #15 and incubated at 37° C for three days.

Culture	Coagulase test read after indicated time in minutes	Dilutions					
		undil.	1:10	1:50	1:100	1:200	1:1000
Staph. aureus strain 15 heated for 75 min. at 56° C	15	-	-	-	-	-	-
	30	-	-	-	-	-	-
	60	++	++	+	-	-	-
	120	+++	+++	++	+	-	-
	overnight 4° C	+++	+++	+++	++	+	-
Staph. aureus strain 15 nonheated	15	-	-	-	-	-	-
	30	-	-	-	-	-	-
	60	++	++	+	-	-	-
	120	+++	+++	+++	++	+	-
	overnight 4° C	++++	++++	+++	+++	++	-

intramuscularly (0.2 ml) with this preparation. The mice were sacrificed after 24, 48, 72 and 120 hours. The muscle tissue was removed, ground in a mortar and pestle, centrifuged and the supernatant tested for coagulase activity. Table 37 shows that coagulase could not be detected under the experimental conditions employed.

TABLE 37

Results of coagulase tests on supernatants prepared from a ground suspension of mouse muscle tissue

Inoculum	Mice sacrificed (hours after inoculations)	Coagulase titer of supernatants
0.2 ml of Staph. aureus #15 heated for 75 minutes in a 56° C water bath	24	negative
	48	"
	72	"
	120	"

DISCUSSION

Mice have been reported to be resistant to staphylococcal infections (Christie et al. 1946). In the experiment to determine the susceptibility of mice of different ages to supernatants prepared from staphylococcal cultures, it was noted that mice in the age range of 6-17 days were very susceptible to these preparations in that death was very rapid. However, it is interesting to note that 18 day old mice, injected with the three day and seven day supernatant, were resistant to these preparations, but that mice 17 days and younger were susceptible. This could probably be due to the mice growing very rapidly during the first sixteen days of life and as a result, are more susceptible. After about sixteen days, growth rate decreases and probably the defense mechanism that makes them resistant is operative. In this age range even one day may make a difference in susceptibility.

Prolonged freezing and freezing and thawing of broth cultures of Staph. aureus had very little effect upon the coagulase titers and toxic activity of broth cultures of Staph. aureus strains #13 and #15. Even after freezing for seven months at -26° C, broth cultures were still toxic for suckling mice and showed coagulase activity. Freezing and thawing a broth culture of Staph. aureus strain #15 several times did

not affect the coagulase titer. Since there are two kinds of coagulase, one a bound coagulase and the other an unbound, (Duthie, 1954) one might expect the bacterial cells to be disrupted during the process of freezing and thawing due to formation of ice crystals thus releasing the bound coagulase and increasing the coagulase titers. However, this was not observed under the experimental conditions employed.

Filtration of the supernatant fluid of a 72 hour broth culture of Staph. aureus strain #15 through different types of filters showed that the Millipore filter did not remove coagulase as did the Mandler and Seitz filters. However, all filtrates were toxic for suckling mice, including those from which coagulase had been removed. The results of this experiment support the suggestion of Menken and Walston (1955) that coagulase is not toxic and that the staphylococcal lesion is due to exotoxins elaborated during metabolic activities of the organisms.

Staphylococci grown in tryptose phosphate broth in the presence of increased carbon dioxide showed an increase in toxic activity as compared to those grown without (added) carbon dioxide. This increase in toxin production was probably due to the pH of the broth culture being maintained below 7.6 and that a higher pH occurs when cultures are incubated without (added) carbon dioxide and this higher pH is detrimental to toxin production (Parker et al. 1926).

In the study on the effect of temperature on coagulase and toxic activity of different strains of Staph. aureus, prolonged incubation of broth cultures in the presence of carbon dioxide favored toxin production; however, the coagulase titers decreased. A close correlation exists between the production of alpha toxin and the production of coagulase (Cowan, 1939) (Christie et al., 1946). However, conditions which are suitable for production of alpha toxin do not favor production of coagulase. Incubation in an atmosphere of carbon dioxide favors production of alpha toxin but is inhibitory to coagulase (Di-Rocco and Fulton, 1939).

Mouse toxicity of broth cultures and Seitz filtrates of strains 5, 13, 15 and 17 was due to toxin production and not coagulase. Cultures of these strains incubated without increased carbon dioxide were not as toxic for suckling mice as were the cultures incubated in the presence of (increased) carbon dioxide. Thus death was more rapid in the mice injected with the increased carbon dioxide preparations.

Cell free filtrates of strains 5, 13, 15 and 17 lacking free coagulase but high in toxin content, were lethal for suckling mice; however, this was not the case with strain #10. This strain was a very poor toxin producer as was noted when the cell free Seitz filtrate prepared from a broth culture incubated in the presence of (increased) carbon dioxide failed to kill suckling mice. However, this was apparently not true

with the other strains. Mouse lethality of this particular strain appeared to be related to the presence of organisms rather than toxin production. It is possible, however, that some strains produce toxin only in vivo. Kleiger et al. (1942) have reported the in vivo production of toxin.

It was noted that strains #15 and #17 were very good toxin producers. The original culture of strain 15 was highly lethal which was not the case with the other strains. Death was not as rapid as it was with the Seitz filtrate of this culture. It appeared that the presence of organisms in this particular culture interfered with the action of the toxin and upon removal of the organisms the toxin reacted much more rapidly.

Strain 17, which was isolated from turkeys, reacted very similar to the human strain 15 with the exception that the original culture containing organisms was not as lethal as was the corresponding culture of strain 15. However, upon Seitz filtration, the filtrate was as lethal as was the filtrate of strain 15.

Boiling of the cell free filtrates obtained from cultures grown in the presence of (increased) carbon dioxide did not affect the activity of these filtrates even after boiling for fifteen minutes. These preparations were very active, especially those obtained from cultures of strains 15 and 17. The original cultures of strain 15 grown in the

presence of (increased) carbon dioxide were still toxic after being boiled for fifteen minutes; however, they required a greater period of time to exert their action. The original culture of strain 17 grown under similar conditions was not as active in that boiling for five minutes inactivated the toxins. The difference in the results was probably due to the fact that the toxins produced by strain 15 were more heat stable than those of strain 17.

Tager (1941) suggested the presence of two fractions of alpha toxin: one a heat labile fraction and the other a heat stable one. The toxins produced by strain 17 are apparently more heat labile and this suggests that probably the heat stable fraction could not exert its effect when the organisms were present. Tager (1941) was able to obtain active preparations after boiling for fifteen minutes if boiled in the absence of inhibitors (intact staphylococci, pepsin and lecithin). With strain 15, in direct contrast with strain 17, the presence of organisms in the culture did not apparently interfere with the action of the toxins as they did with strain 17; however, it required a greater period of time for the toxins to exert their effect. Both strains, however, yielded very active preparations upon removal of the organisms by Seitz filtration. Boiling these preparations for fifteen minutes did not render them inactive, indicating that the toxins were heat stable and more active in the absence of intact organisms. These results

suggest that the lethality for suckling mice is correlated with toxin production rather than with coagulase.

In the experiment to determine if the heat labile and the heat stable fractions of toxin were immunologically different, no significant difference was shown. In addition no significant protection was afforded when mice were injected with a mixture of filtrate and homologous antiserum.

The supernatant fluid prepared from a suspension of ground muscle tissue obtained from mice previously inoculated with a heat treated suspension of Staph. aureus strain 15 yielded no detectable coagulase under the experimental conditions employed. The suspension was rendered coagulase negative by heating for 75 minutes in a 56° C water bath. However, this treatment did not destroy the ability of the organisms to produce coagulase. A titer of 1:200 was obtained with a broth culture of this heat treated suspension; however, when mice were inoculated with this suspension, coagulase was not detectable suggesting that either it was not produced in vivo or that it was produced in amounts not detectable by the usual procedures employed in coagulase testing.

SUMMARY

Mice in the age range of 6-17 days of age were found to be susceptible to the injection of staphylococcal culture supernatants. Older mice were found to be resistant.

Freezing and thawing of a broth culture of Staph. aureus strain 15 had no effect upon the coagulase or toxic activity, even after seven months at -26° C.

Filtration of broth cultures through Seitz and Mandler filters removed coagulase but had no effect upon the toxic activity. Millipore filtration did not remove coagulase.

Mouse toxicity of broth cultures and Seitz filtrates of Staph. aureus strains 5, 13, 15 and 17 was due to toxin production rather than coagulase. Cultures grown in the presence of increased carbon dioxide were more toxic for suckling mice than were the ones grown without added carbon dioxide.

Mouse lethality of Staph. aureus strain 10 appeared to be related to the presence of organisms rather than alpha toxin.

Cell free filtrates of strains 5, 13, 15 and 17 were more lethal for suckling mice than were the original cultures which contained organisms.

In addition boiling of Seitz filtrates of strain 15 and 17 did not destroy the toxic activity of these preparations whereas it did with strains 5 and 13.

Strain 17, a turkey isolate, resembled the human strain 15 in its toxic activity. Seitz filtrates of both strains were more toxic than the original cultures.

No immunological difference was shown between the heat labile and the heat stable fractions of alpha toxin. In addition no significant protection was obtained when mice were injected with a mixture of filtrate plus homologous or heterologous antiserums.

Coagulase could not be detected in the supernatant of a suspension of ground mouse tissue under the experimental conditions employed.

Heating a suspension of Staph. aureus strain 15 for 75 minutes in a 56° C water bath rendered the suspension coagulase negative; however, the ability of these organisms to produce coagulase was not destroyed.

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STUDIES ON STAPHYLOCOCCUS AUREUS WITH REFERENCE TO COAGULASE ACTIVITY

by

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ABSTRACT

As observed in these studies, mice in the age range of 6-17 days of age were more susceptible to the injection of staphylococcal supernatants than were older mice.

Freezing and thawing and storage of a staphylococcal broth culture at -26° C for seven months did not affect the coagulase titers nor the toxic activity of this culture.

It was observed that filtration of staphylococcal broth cultures through Seitz and Mandler filters removed coagulase, but did not affect the toxic activity of these cultures. Millipore filtration did not remove coagulase.

Prolonged incubation of Staph. aureus cultures in the presence of increased carbon dioxide favored the production of toxin rather than coagulase.

Lethality for suckling mice was due to the presence of staphylococcal exotoxin in the cultures and in cell free filtrates rather than coagulase. In addition, cell free filtrates were more lethal than were the cultures which contained organisms.

Exposure to boiling temperature of cell free filtrates, prepared from cultures of Staph. aureus strains 15 and 17, did not destroy the toxic activity of these filtrates as it did with the filtrates of strains 5 and 13.

The results obtained with strain 17, a turkey isolate, resembled those of the human strain 15. Cell free filtrates of both strains were quite toxic; however, the toxins of strain 15 appeared to be more heat stable than those of strain 17. No significant immunological difference was shown between the heat labile and the heat stable fractions of staphylococcal exotoxin. In addition, mice injected with a mixture of cell free filtrates plus homologous or heterologous antiserum were not protected from the toxic activity of these filtrates.

A positive coagulase test was not obtained with a suspension of Staph. aureus strain 15 heated at 56° C for 75 minutes. However, this treatment did not destroy the ability of this organism to produce coagulase.

In the experiment to determine if coagulase is produced in vivo, coagulase could not be detected in the supernatant fluid of a suspension of ground mouse muscle tissue.

RESEARCH PROPOSALS

submitted

by

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in partial fulfillment of
the requirements for the
degree of

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Department of Microbiology

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RESEARCH PROPOSALS

1. Coagulase production has been customarily taken as an *in vitro* reaction indicating potential pathogenicity of *Staph. aureus*; it is proposed to determine if coagulase is produced *in vivo*.

2. Coagulase is produced in many different types of media; experiments should be carried out to determine necessary conditions and types of media for maximum production of coagulase.

3. The pathogenicity of *Staph. aureus* has been attributed to many metabolic products sometimes termed "virulence factors;" no conclusive statements of significance can be made implicating any single factor. Correlated studies are proposed to compare the "virulence factors" of several strains of *Staph. aureus*.

4. Several strains of *Staph. aureus* are penicillin resistant; studies are proposed to compare coagulase and alpha hemolysin production of resistant and nonresistant strains.

5. Several exotoxins such as alpha, beta and delta hemolysins are produced by *Staph. aureus* and have been implicated in the virulence mechanism of this organism. Studies are proposed to determine if other unidentified toxins are produced, and if so, to study their characteristics.

6. Human beings are the main reservoir of Staph. aureus; however, some strains can be isolated from animal sources. Studies are proposed to compare human and animal strains with regard to coagulase and toxin production.

7. It is proposed to study the effect of physical and chemical inactivation of purified and concentrated coagulase.

8. Studies are proposed to determine the physiological effects of highly purified coagulase.

9. Many people are carriers of encapsulated and nonencapsulated strains of Staph. aureus. Studies are proposed to compare the characteristics of these strains with strains isolated from staphylococcal lesions to determine if there are any differences in their virulence mechanisms.

10. The effects of ionizing and nonionizing radiation upon the production of coagulase and toxins of encapsulated and nonencapsulated strains of Staph. aureus should be investigated.